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Award Number: DAMD17-03-1-0601

TITLE: Integrin-dependent Antagonism of Tamoxifen Therapy Through Transcriptional Replacement: Establishing a Basis for a New Combined Therapy

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REPORT DATE: August 2004

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2004	Final (1 Aug 2003 - 31 Jul 2004

4. TITLE AND SUBTITLE
Integrin-dependent Antagonism of Tamoxifen Therapy Through Transcriptional Replacement: Establishing a Basis for a New Combined Therapy

5. FUNDING NUMBERS
DAMD17-03-1-0601)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 words)*** *(abstract should contain no proprietary or conficential information)*

Accruing evidence suggests that integrin-dependent cell attachment signaling, and estrogen hormonal response are closely interconnected. For example, studies by us and others of the BCAR proteins (BCAR1 and BCAR3: (1, 2)) have indicated that these proteins physically associate with each other, and function both in signal transduction relevant to integrin stimulation, and in mediation of Tamoxifen (Tam) resistance. The goal of this proposal was to explore the interrelationship between integrin signaling, cell attachment status, and Tam resistance. A specific hypothesis was that activation of integrin signaling reduces the Tam-dependent inhibition of essential estrogen-dependent transcription. In particular, the proposal sought to explore how the formation of organized three-dimensional structures (spheroids) by metastasizing tumor cells, which greatly enhances their resistance to treatment with a number of drugs (reviewed in (4), might modulate Tam resistance and the estrogen-dependent transcriptional program. As described below, we found that spheroids unexpectedly did not result in increased Tam resistance. However, we did find that manipulation of expression of BCAR1 resulted in changes in the transcription of estrogen-regulated genes, suggesting the initial hypothesis of an integrin-estrogen connection at the level of transcription is worth further investigation.

14. SUBJECT TERMS
tamoxifen, integrin, BCAR1, transcription

15. NUMBER OF PAGES
13

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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FINAL REPORT, BC023939. Integrin-dependent antagonism of tamoxifen therapy through transcriptional replacement: establishing a basis for a new combined therapy.

INTRODUCTION. This project was intended to study the interrelation between estrogen response and cell microenvironment. In excess of 65% of breast tumors express the estrogen receptor (ER)-alpha protein, which is activated by binding estrogens, moves to the cell nucleus, and turns on the transcription of genes whose function contributes to tumor progression. Tamoxifen (Tam) antagonizes the ability of estrogens to bind ER-alpha, resulting in the inhibition of estrogen-dependent genes. Tam therapy is a standard treatment for ER-alpha-positive breast cancers. However, up to 50% of ER-alpha positive tumors do not respond well to Tam. We wished to gain insight into the process of Tam resistance in these tumors.

The **specific hypothesis** we chose to address was based on the fact that accruing evidence suggested that integrin-dependent cell attachment signaling, and estrogen hormonal response are closely interconnected. For example, studies by us and others of the BCAR proteins (BCAR1 and BCAR3: (3, 7)) have indicated that these proteins physically associate with each other, and function both in signal transduction relevant to integrin stimulation, and in mediation of Tam resistance. The means by which integrin signaling is protective for Tam treatment has not been established. However, an attractive hypothesis stems from the long established observation that transcriptional programs can be greatly affected by the status of cell shape and cell-cell attachments (e.g. (6)). For instance, formation of organized three-dimensional structures (termed spheroids or globular microlumens) by metastasizing tumor cells greatly enhances their resistance to drug treatment (reviewed in (10)). Hence, formation of organized three-dimensional structures would be predicted to limit the ability of Tam to induce down-regulation of estrogen-dependent transcription.

The objective of the proposal was to test the idea that the formation of integrin-dependent, three-dimensional spheroids reduces the Tam-dependent regulation of estrogen-dependent transcription. This work was broken down into several different tasks. These were, Task 1. To develop a baseline of growth parameters for two estrogen-receptor positive cell lines, MCF-7 and ZR-75 cells. This would involve plating the cell lines under different growth conditions that would differentially affect integrin signaling, and optimizing timing of plating and drug concentration so as to achieve a good Tam effect (cytostasis, cell death) in cells grown on plastic (i.e., without extensive integrin engagement, or 3D organization. This would allow us to determine an optimal time for mRNA harvest, to perform the subsequent task (Task 2), which was to measure change in ER-alpha dependent transcription in response to cell culture (i.e., integrin ligation) conditions (Months 3-8), using microarrays and bioinformatics to interpretation results. Finally, a complementary Task 3 was to explore the mechanism of integrin-dependent inhibition of Tam-dependent transcriptional effects. This would be done by using various approaches to block integrin signaling, and determining if this restored a transcriptional pattern to resemble.

BODY. This is a ***revised*** final report. We apologize for the abbreviated nature of the last report: because of miscommunication with our grants office, we supplied a final report in a format for an NIH RO1 short summary. In this much expanded revision, we not only provide a much more detailed discussion of the project, but also explicitly respond to the comments made by the reviewer of the initial, short, final report.

Task 1. Matrices. The first step was to establish differing conditions of cellular microenvironment that might affect integrin signaling. In this effort, there were two scientific issues. The first one was the physical constraints provided by a fibrillar matrix. The second was the provision by the microenvironment of specific ligands that would differentially activate individual integrins: for instance, laminin activates integin alpha3beta1, while collagen preferentially activates alpha2beta1. Key studies demonstrating the protective effect of growth in three dimensional microenvironment frequently used Matrigel, which is a complex mixture of multiple components, including laminin, collagen IV, heparan sulfate proteoglycans, and other factors (11). We wished to thoroughly address the importance of each factor. Hence, for growth conditions, we considered growth on tissue culture plastic; matrigel; collagen; collagen plus laminin; and collagen spiked with Matrigel. For experiments with Matrigel, we also compared protocols in which cells were seeded into a molten Matrigel, as opposed to spread on top of Matrigel (looking ahead to the desire to extract mRNA, we thought this would be facilitated if cells were closer to the surface of the matrix support, and some protocols suggested seeding cells on top of matrix resulted in sufficient infiltration of cells into matrix to obtain biological effect (5), while making them easier to extract.

Cell lines. The second step was the optimization of timing of growth of cell lines in the media. In the papers providing the basis for this study, characteristic differences in growth response to culture on plastic versus Matrigel were observed between cancer cell lines, and cells that maintained epithelial cell characteristics (discussed in (10)). For the latter cells, the ability to form polarized structures played an important role in resistance to apoptosis. For full comparison of effects between transformed and minimally transformed cells, besides using the MCF-7 and ZR-75 cell lines originally proposed in the Concept award, we also used MCF-10F and MCF-10A cells. Although these cells are ER-alpha negative, at minimum MCF-10F are nevertheless estrogen- and tamoxifen-responsive, probably at least in part due to action on ER-beta (for instance, see (12, 14)). MCF-10A cell lines are the major model line used for study of breast cell polarization in 3D culture systems. Beyond these, there are very few relatively normal cell lines to choose from. In plating the cells, we separately evaluated plating of cells into medium already containing Tam, versus allowing cells to first acquire a spheroidal 3D structure (testing time periods ranging from 5 days to 12 days, as discussed in (4, 10, 17)).

Tamoxifen. For these studies we used standard conditions for cell culture, using DMEM medium (MCF7 and ZR-75) or high calcium medium (MCF10F) with charcoal stripped serum, supplemented with 10 nM estrogen, and concentrations of Tam ranging from 0 to 25 μM for initial evaluation. We found that a concentration of 20 μM induced inhibition of proliferation (as measured by Alamar Blue staining) within 2-3 days after

addition. We also performed some studies comparing Tam with 4-hydroxytamoxifen (OHT), and found no significant difference for the parameters we assayed.

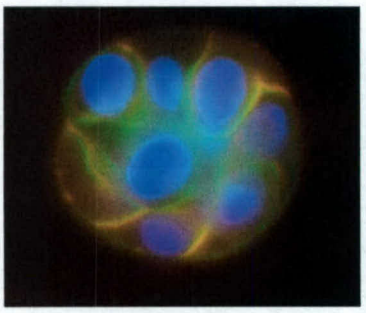

Figure 1. MCF-7 cells 5 days after growth in Matrigel: other cells look equivalent. Blue, DNA; green, paxillin; red-E-cadherin.

Results. We assessed the three cell lines to establish growth profile in the different matrices. In cells that had not been challenged with Tam, over the course of 2 weeks, cells grown in matrices gradually grew in microcolonies. Our data were consistent with the published literature. Initially, all cells grew in non-distinguishable clusters, with MCF7, ZR-75, MCF-10A and MCF10F cells comparable for the first several days in matrix (Figure 1). However, by 10 days of culture in Matrigel, "normal" MCF-10F cells formed round, even spheroids, in which the cells in the center of the spheroid were removed by apoptosis. Transformed MCF7 and ZR-75 cells formed uneven clumps of cells, with no observable cell death or "hollowing out" of the center to reconstitute a polarized lumen. Phase micrographs and staining with integrin make this point clearly (Figure 2): we also stained cells with other markers to confirm appropriate polarization (not shown). Also paralleling the literature, MCF-10F cells grown on Matrigel formed clear hollowed and organized spheroids, while the same cells grown on collagen were much more disorganized (Figure 3). Based on these results, we believed we had correctly established cell growth conditions.

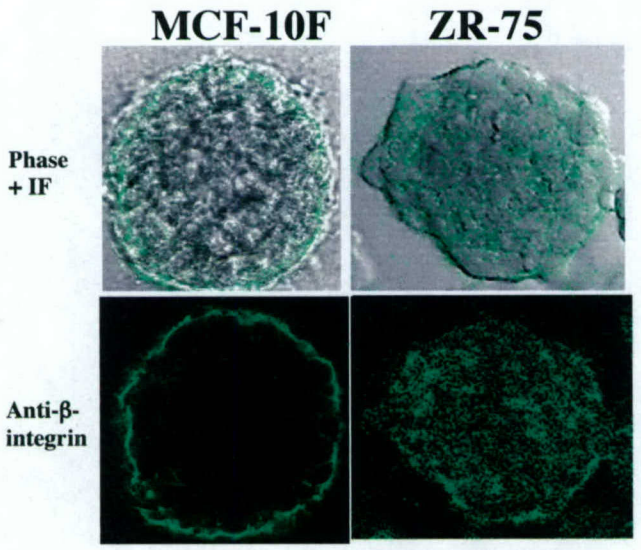

Figure 2. Growth of immortalized untrans-formed MCF-10F versus transformed ZR-75 cells in Matrigel, after 10 days of culture in matrix.

We next assessed the growth of the cells grown on plastic or on different matrices following treatment with Tam. A representative experimental set-up is shown in Figure 4. Cells were grown in 96 well plates; 4 independent wells were used for each combination of matrix and Tam. All experiments were repeated at least 3 times. As shown in Figure 3, the results of these experiments initially surprised us. With Alamar blue staining, bright red color is reflective of viable cell numbers, with growth inhibition reflected by a transition through purple to blue (cytostasis, death). Based on these results, the cells grown on Matrigel and collagen appeared to be more sensitive to Tam than did cells grown on plastic. Comparable results were obtained in the three different cell lines we examined. This was extremely puzzling, because our anticipation based on the literature was that growth in Matrigel or other support matrices would either have a

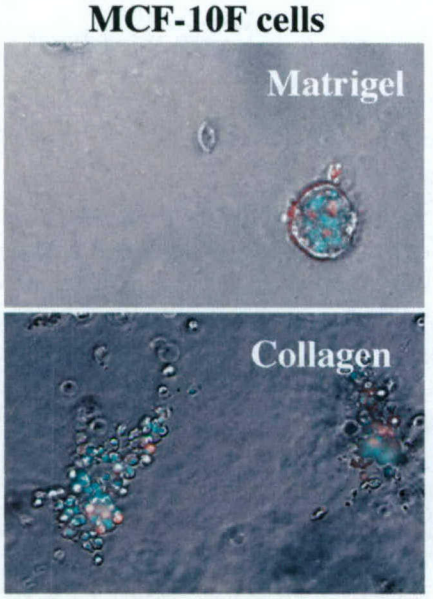

Figure 3. Effect of matrix composition on the organization of MCF-10F cells

protective effect, or no effect; we did not anticipate a sensitization to Tam. Cells were comparably affected by Tam under all matrix growth conditions (Figure 5). We also note that MCF-10F cells lines behaved similarly to MCF7 and ZR-75 in their response to Tam (Figure 6), indicating the presence of ER-alpha was not essential for this proliferations response (again, paralleling published literature, (12)).

We continued exploring different growth conditions to understand this finding. The result of extensive probing of conditions is summarized in the following Figure 7. In comparison of the proliferation rate of cells, it is clear that several days after initial seeding, the growth rate of cells on matrices slows in comparison to cells grown on plastic. Although this is masked with cells plated at standard cell densities (optimal for imaging, and rapid development of Alamar blue signal), it can be seen with cells plated at low densities with long development times: This is visible both by Alamar blue staining (where untreated matrix grown cells are blue or purple in comparison to red plastic grown cells), and by observed acidification of the culture medium (red with matrix grown cells, but more yellow with plastic grown cells). Because of this difference (which presumably reflects either reduced proliferation due to differentiation of cells in matrix, or alternatively, the limited ability of cells within spheroids to proliferate), cells growing in matrix are still in a linear range facilitating detection of Tam effects, while cells growing on plastic are approaching a saturating range. As a result, Tam inhibition of cell proliferation is masked. With final optimization of conditions, so that the number of cells at the time of assay 2-3 days after Tam treatment would be identical, there was

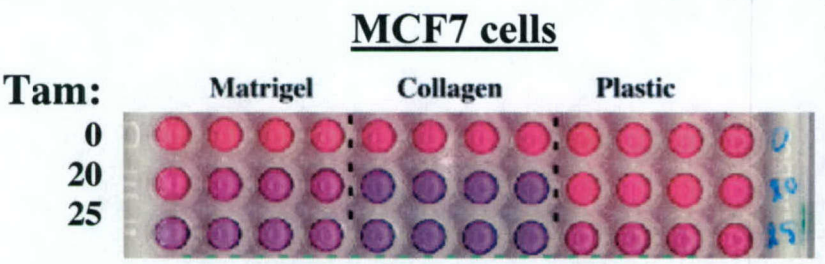

Figure 4. Growth of MCF7 cells in Matrigel, Collagen, and on plastic, with or without Tam.

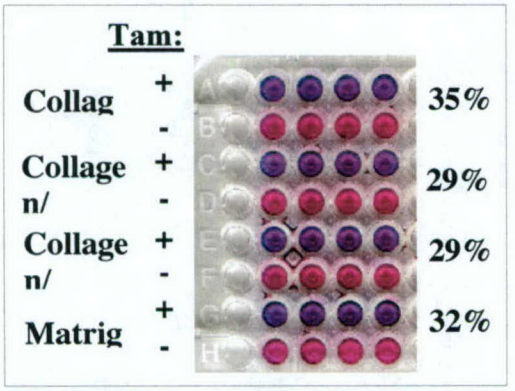

Figure 5. Cells were pregrown on the indicated matrix (left), cell number scored by Alamar blue, then treated for 2 days with 25 mM Tam, or left untreated. Percents(right) represent quantitation by platereader of relative cell numbers in Tam + versus - samples.

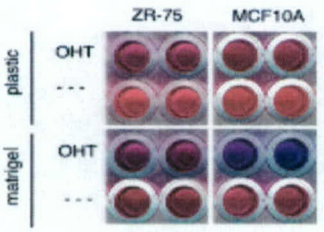

Figure 6. ZR-75 or MCF-10F cells grown on plastic or on Matrigel, with or without OHT.

no longer any selective effect of growth in matrix. Tam inhibited the growth of the cells at the equivalent rate. The reviewer of the first abbreviated final report suggested we examine cytotoxic agents. This experiment was not proposed in the original proposal, and given the negative results of the primary experiment, under conditions in which cells were growing according to described parameters (Figures 1-3), this seemed inappropriate.

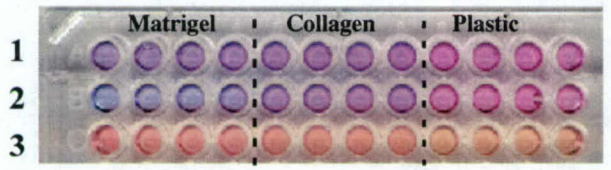

Figure 7. MCF7 cells grown without (1, 3) or with (2) Tam. Rows 1 and 2 are stained with Alamar blue; Row 3 is unstained media(with cells growing in Matrigel, in collagen, or on plastic, as indicated).

With this finding, the basis for the second task was eliminated: as growth in neither defined matrix component nor Matrigel in any way inhibited the toxic effect of Tam, it seemed a waste of reagents, and effort to look for differences in estrogen-responsive genes under the different growth conditions. Moreover, with each cycle of experimentation taking over two weeks, it took considerably longer than anticipated to work through these optimization steps, limiting the time left within the one year concept award. We therefore chose to focus our efforts on the experiments outlined in Task 3,

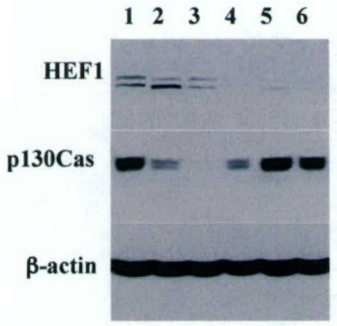

Figure 8. Control scrambled siRNA (1), or siRNA targeted to p130Cas (2,3) or HEF1 (4-6): Western analysis. Beta-actin is a loading control.

Task 3. In this task, we proposed to directly manipulate integrin signaling, and examine consequences for expression of canonical estrogen-regulated genes. Because of the surprising results of Task 1, we wished to be sure that we were efficiently detecting estrogen-regulated genes. BCAR1 and the interacting protein BCAR3 were directly connected both to integrin signaling and to regulation of Tam response (1, 7, 15, 16). BCAR3 (also termed AND-34) physically interacts not only with BCAR1 (also termed p130Cas), but with HEF1 (3, 9). Together, p130Cas and HEF1 define the Cas family, much studied by our group (13). p130Cas is ubiquitously expressed: HEF1 is primarily restricted to lymphoid and epithelial cells, with the latter relevant to breast cancer. To provide the best chance of detecting a measurable estrogen-relevant transcriptional response following manipulation of integrin signaling, we have looked at cells with up- and down-regulated p130Cas and HEF1. To do so, we evaluated panels of siRNAs to deplete p130Cas and/or HEF1, picking those that were most effective (Figure 8). We also used overexpression cell lines based on MCF7 parental cells, in which a tetracycline-regulated promoter would control induction of HEF1 or p130Cas (e.g., Figure 9). For each experiment, we routinely used Western analysis to confirm effective depletion or induction of p130Cas and/or HEF1. We note, although we had only proposed in the original Concept award to use approaches to inhibit

integrin signaling, we agree with the reviewer of the first final report that it made sense to look both for up and down regulation of BCAR1 and HEF1, and have done so. We note, we are now out of the time period of the Concept award, and this work is ongoing., so final conclusions as summarized in this report are likely to change and expand in the next few months.

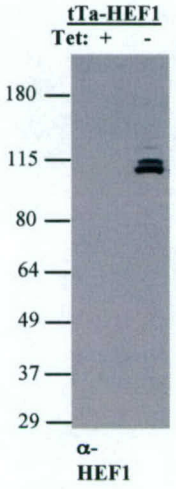

Figure 9. Induction of HEF1 in MCF-7 cells, detected by monoclonal antibody. Film lightly exposed to emphasize degree of induction.

Our ultimate goal, expressed in the proposal, was to look at microarrays. Because there can be technical complications to the use of microarrays (for instance, some mRNAs of particular interest may be of low abundance, and not detectable), and because the results of Task 1 made us unsure whether any effect was to be anticipated, we used a more conservative initial approach. Fox Chase Cancer Center has established a quantitative PCR facility under the direction of Drs. Anthony Yeung and Emmanuelle Nicolas. This facility optimized primers to perform direct amplification of a number of a number of transcripts known to be estrogen regulated. In addition, we assessed a number of transcripts linked to the process of epithelial-mesenchymal transition, important for breast cancer progression; and some transcripts we thought were "irrelevant" to estrogen signaling, to act as standards for variation seen. We have run about 20 experiments. We have spent some time exploring timing for this analysis (24, 48, or 72 hours after depletion). One representative table is shown (Table 1; at end of proposal body). It is clear that at least some estrogen-regulated transcripts, such as CCN2, are negatively regulated by p130Cas and HEF1. Other transcripts related to breast cancer metastasis, such as S100A4, and FN1, also show negative regulation by these proteins. Others, such as the estrogen-regulated pS2, and heparanase, appear to be positively regulated by the Cas proteins. The effects seen are reproducible: the magnitude of effects is generally in the order of 2-3 fold, although in some cases (i.e. for S100A4) a more dramatic effect is seen. Clearly, these proteins do have the potential to affect the level of estrogen-regulated and other transcripts. It is our intent to move these studies to microarray work over the next few months, using a 48 hour timepoint after BCAR1 or HEF1 manipulation.

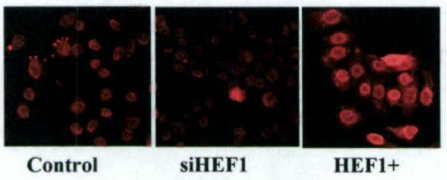

Figure 10. HEF1 controls nuclear localization of Snail. Shown, MCF7 cells with empty vector (control), or with vector expressing siRNA to HEF1 (siHEF1) or full length HEF1 cDNA (HEF1+), in each case in induced, tetracycline-minus. Snail is indicated in red.

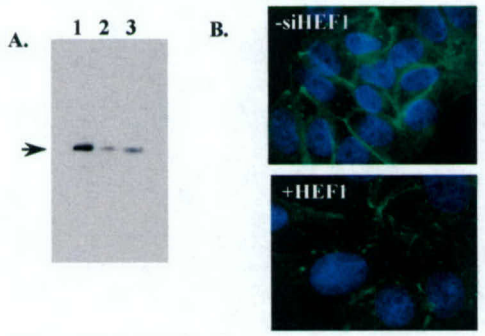

Figure 11. A. E-cadherin protein expression in cells with siRNA-depleted HEF1 (1), overexpressed HEF1 (2), or control MCF7 cells (3). **B.** E-cadherin (green) staining of cells with depleted HEF1 (top) or over-expressed HEF1 (bottom)

Transcripts	Tet-Vector (72h)	Tet-Cas (72h)		siCas (72h)	
Estrogen related					
IGF1R	1	0.78	0.87	1.70	1.44
IGF2R	1	1.40	1.18	1.07	0.78
TFF1/PS2	1	3.0	2.7	1.1	
A1B1/NCOA3	1	0.96	1.20	0.85	0.86
CYP19A1	1	1.02	0.45	2.69	0.21
CYR61/CCN1	1	1.54	1.61	2.69	2.54
CTGF/CCN2	1	0.26	0.26	2.82	1.69
EMT-related					
E-Cadherin	1	1.19	1.3	0.57	0.68
snail	1	0.87	0.90	2.33	1.89
OCLN	1	1.45	1.47	0.9	1.41
Claudin7	1	1.06	1.31	0.73	0.84
MSF	1	0.48	0.88	2.4	2
MUC1	1	0.25	0.29	0.86	0.96
FN1	1	0.72	0.84	4.65	3.42
Related					
FSP1/S100A4	1	0.15	0.10	1.42	0.9
PDZK1	1	1.04	1.16	0.5	1.08
ESR1	1	0.44	0.71	0.46	0.62
NOV	1	0.88	2.09	1.56	1.33
HIF1-1delta11	1	1.43	1.17	1.41	1.21
SPARC	1	0.2	1.57	3.2	1.8
Hepararanase	1	1.38	1.4	0.5	0.64
Ajuba	1	0.96	1.15	0.67	0.79
Aurora A/STK6	1	1.19	1.45	0.96	1.06

Table 1. Each column represents an independent experimental run with samples in duplicate. Red: Reduction in transcript levels. Green: Increase in transcript levels. Black: basal level of transcript levels (~ 1), and no significant or consistent change in transcript levels versus "basal, which is the transcript level in cells expressing empty vector, assigned an arbitrary value of 1.

An important question is whether the effects seen are direct or indirect. Intriguingly, in the past year, one paper has appeared indicating that p130Cas/BCAR1 directly associates with ER-alpha (2) to modulate non-genomic effects of estrogen. This would argue in favor of an indirect mechanism, as would the cytoplasmic localization of both BCAR1 and BCAR3: however, the question needs further investigation. There may also be a role for more direct control of estrogen transcription. One recent publication has described a connection between estrogen-signaling and cell-adhesion signaling in metastatic breast cancer based on the function of the Snail repressor protein (8). In this study, it is shown that absence of estrogen signaling causes upregulation of Snail, a promoter of epithelial-to-mesenchymal transition and metastasis. In ongoing work in our laboratory and with collaborators, we have found that HEF1 controls the nuclear accumulation (activity) of Snail (Figure 10). Snail represses the transcription of E-

cadherin. We have found that manipulation of HEF1 (Figure 11) and p130Cas (not shown) in each case regulates the accumulation of E-cadherin, an important marker of tumor aggressiveness, and one also known to be affected by estrogen regulation. Our laboratory is continuing to investigate this signaling axis.

KEY RESEARCH ACCOMPLISHMENTS.

- We have determined that growth of cells as spheroids in culture does not result in increased resistance to tamoxifen.
- We have found that manipulation of BCAR signaling causes changes in the transcription of estrogen-regulated target genes, as well as in other genes.
- We have implicated BCAR1/HEF1 signaling through the Snail transcriptional repressor as a mediator of the estrogen-integrin transcription connection.

REPORTABLE OUTCOMES. Some of the work described in this proposal has been included in an abstract was presented at the American Society of Cell Biology meeting in December 2004 in Washington, D.C. It has also been included in an RO1 application (CA113342) submitted June 2004 to the National Institutes of Health.

CONCLUSIONS. This study has shown that growth in three dimensions does not influence the ability of Tam to inhibit cell growth. This study has supported the idea that the BCAR proteins, important effectors of integrin signaling that also confer tamoxifen resistance, can regulate at least some estrogen-dependent transcription. However, the p130Cas and HEF1 effects at the level of transcription are not enormous, suggesting that other non-genomic effects leading to resistance to tamoxifen perhaps by increasing overall resistance to apoptosis may be involved. Nevertheless, the ability of these proteins to regulate Snail and E-cadherin indicates that they may at least indirectly communicate with transcriptional machinery known to be important for breast cancer development.

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APPENDICES. None.